Embryogenesis and Development of *Epimenia babai* (Mollusca Neomeniomorpha)

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Abstract. Neomenioid aplacophorans (= Solenogastres) constitute one of the main lineages of molluscs. Developmental data of early embryogenesis and larval development of neomenioids are available for some species based on histological sections. I used other techniques to study the development of *Epimenia babai* Salvini-Plawen, 1997, and here I report new data on neomenioid development. The embryos of *E. babai* are lecithotrophic and cleavage is spiral, unequal, and holoblastic. Two polar lobes are formed, one at the first cleavage stage and one at the second cleavage stage. No evidence of external metameric iteration is visible through scanning electron microscopy or histology at any stage. A ciliated foot, a pedal pit, and aragonitic spicules develop from the definitive ectoderm. A spicule begins as a solid tip, continues to an open-ended hollow spicule, and finally becomes a closed-ended hollow spicule.

The free-swimming trochophore larvae of *E. babai* have been considered unusual in lacking the characteristic neomenioid cellular test, an outer locomotory structure within which the entire definitive adult body develops. However, through the use of scanning electron and light microscopy, semithin sections, Hoechst nuclear staining, and programmed cell death staining to study the ontogeny and fate of the apical cells, I show that the entire pre-oral sphere (the apical cap) of the larvae is similar to the test of the other neomenioids. The results suggest that the test of the neomenioid larvae is an enlarged pre-oral sphere of a trochophore. The test morphologies of neomenioid larvae are compared to those of pericalymma larvae of protobranch bivalves, and the homology and evolution of molluscan larval tests is discussed.

Introduction

Neomenioid aplacophorans (= Solenogastres) are shell-less vermiform molluscs with a ciliated foot and an epidermal cuticle covered by many calcareous spicules. Given that aplacophorans are hypothesized to hold a basal position among molluscs (Götting, 1980; Salvini-Plawen, 1985; Salvini-Plawen and Steiner, 1996; Ivanov, 1996; Waller, 1998; Haszprunar, 2000), their development may provide the key to answering outstanding questions in the evolution of molluscan larvae. However, little is known about the developmental processes and features of neomenioid aplacophorans. Larval descriptions are based on five neomenioid aplacophoran species: *Epimenia babai* Salvini-Plawen, 1997 (as *E. verrucosa*); *Halomenia gravida* Heath, 1918; *Neomenia carinata* Tullberg, 1875; *Nematomenia* (= *Dondersia*) *banyulensis* Pruvot, 1890; and *Rhopalomenia* (= *Proneomenia*) *aglaopheniae* Kowalewsky and Marion, 1887 (Pruvot, 1890, 1892; Heath, 1918; Baba, 1938, 1940, 1951; Thompson, 1959, 1960). Early cleavage pattern is known only for *E. babai* (Baba, 1940, 1951; and herein). Nothing has been reported on the early embryology of chaetodermomorph aplacophorans (= Caudofoveata), and their larval descriptions are based on a drawing of *Scutopus robustus* (Salvini-Plawen, 1990), on drawings based on Gustafson's unpublished sketches (Nielsen, 2001), and on scanning electron micrographs of *Chaetoderma nitidulum* larva (Nielsen, presented at World Congress of Malacology 2002).

Neomenioid aplacophorans are hermaphroditic and either brood juveniles (Heath, 1918; Salvini-Plawen, 1978) or have free-swimming lecithotrophic larvae with an outer locomotory larval test, within which the definitive adult structures develop (Pruvot, 1890, 1892; Thompson, 1960). A similar enveloping test, or "pericalymma," has also been described for the larvae of protobranch bivalves (Drew, 1897, 1899a, b, 1901; Gustafson and Reid, 1986, 1988a, b; Gustafson, 1987; Gustafson and Lutz, 1992; Zardus and

Morse, 1998). The homology of larvae with tests among protobranch bivalves and neomenioid aplacophorans remains uncertain because the phylogenetic relationships of these groups are still debatable, the morphology of these larvae is quite diverse, and the morphology of the neomenioid test is poorly understood (Pruvot, 1890, 1892; Drew, 1899b; Gustafson and Reid, 1986; Thompson, 1959, 1960; Gustafson and Lutz, 1992; Zardus and Morse, 1998).

The neomenioid aplacophoran *Epimenia babai* from Japan is considered to have a unique larval form among neomenioid aplacophorans in that it lacks the true test structure characteristic of neomenioid pericalymma larvae (Baba, 1938, 1940, 1951, 1999; Thompson, 1960; Nielsen, 2001). Baba (1938, 1940, 1951) was the first to describe the larval development of *E. babai*, and he suggested that the morphology and fate of the apical cells of the species do not resemble the pericalymma test cells of other neomenioid aplacophorans. Because of its unique development, the larval form of *E. babai* has been suggested to be an intermediate form between pericalymma and trochophore larvae (Baba, 1938, 1940, 1951, 1999) called "stenocalymma" by Salvini-Plawen (1973, 1980). The descriptions by Baba were based primarily on histological sections, but details of morphology and cell fate during embryogenesis were not studied, and thus such differentiation is not certain.

Adult specimens of *E. babai* are easily collected and maintained in the laboratory. Whereas most aplacophorans are small (1–5 mm) and inhabit the deep sea where they are not easily obtainable for developmental studies, *E. babai* can reach 30 cm in length and lives in relatively shallow water from 20 to 70 m in depth (Salvini-Plawen, 1997). The embryos are relatively large (*ca.* 250 μm) and can be maintained at room temperature. Taking advantage of the accessibility of *E. babai*, this study reexamines the early and late development of the species to elucidate some of the unknown developmental features of a neomenioid aplacophoran, such as the morphology and fate of the pericalymma test.

Materials and Methods

Animal collection and culture

Adult specimens of *E. babai* were collected offshore near Amakusa Marine Biological Laboratory (Kumamoto, Japan) in 1999 and 2000 during the most active breeding period between June and September (Baba, 1938, 1940, 1951). These specimens were up to 20 cm in length and 1 cm in diameter. The exemplars were collected from synthetic gill nets (3 m width, 500 m length, 5 × 5 cm mesh size) that were set on a rocky bottom at a depth of 20 to 30 m among beds of the soft coral *Alcyonium gracillimum*.

The adults were maintained in an aquarium with running seawater at ambient temperature, about 26 °C, and were supplied with fresh *Alcyonium gracillimum* for food. The animals spawned spontaneously several hours after sunset or were induced to spawn by turning off the light after 12–14 h of constant light. Spawned egg sheets (see Fig. 1a) were transferred to culture jars containing filtered seawater (0.45-μm mesh) with streptomycin (50 mg/ml) and penicillin (35 mg/ml) to reduce bacterial growth (Strathmann, 1987). Cultures were kept at both ambient temperature and 16 °C, and their development was compared. Developmental descriptions are based on cultures maintained at 16 °C. More than 1000 embryos from three adults were analyzed.

Scanning electron microscopy

Egg capsules were freed from a jelly mucous casing by gentle agitation, and the embryos were dissected out of their capsules with fine forceps. The embryos were fixed in 1% osmium tetroxide (OsO_4) in filtered seawater and washed twice in 0.2 M sodium cacodylate buffer solution (pH 7.4). They were then dehydrated through a series of ethanol washes (20%, 50%, 75%) and transported from Amakusa to Woods Hole, Massachusetts, where dehydration in ethanol (100%, 100%) was completed. The samples were critical-point-dried (SAMDRI-780), mounted on aluminum stubs with double-sided tape, coated with gold in a sputter-coater (SAMSPUTTER-2a), and imaged using a JEOL scanning electron microscope (JSM-840).

Semithin sections

Dissected embryos were first fixed in a solution of 3% gluteraldehyde in 0.2 M sodium cacodylate buffer at pH 7.4, washed in 0.2 M sodium cacodylate buffer, postfixed in 1% osmium tetroxide (OsO_4), and washed in the buffer again. The embryos were then dehydrated, transported as above, embedded in Araldite/Epon (EMbed-812), and sectioned with glass knives into semithin sections (0.30–1.0 μm). The sections were mounted onto clean slides on a hot plate (60 °C), stained with a mixture of methylene blue and azure II (Richardson *et al.*, 1960), washed under tap water, and imaged on a Zeiss light microscope.

Hoechst nuclear staining

Larvae were incubated overnight at 4 °C in Hoechst solution (1 μg/ml) in 4% formalin (in seawater). The larvae were then washed in 1× phosphate buffered saline (PBS), mounted on a glass slide, and examined with fluorescence under a microscope with a UV filter set.

Programmed cell death determination

Cell viability and apoptosis in the larvae were detected *in vivo* using acridine orange/ethidium bromide staining according to the method described by Martin *et al.* (1996) with slight modifications. Programmed cell death (PCD) occurs frequently during animal development when larval tissues
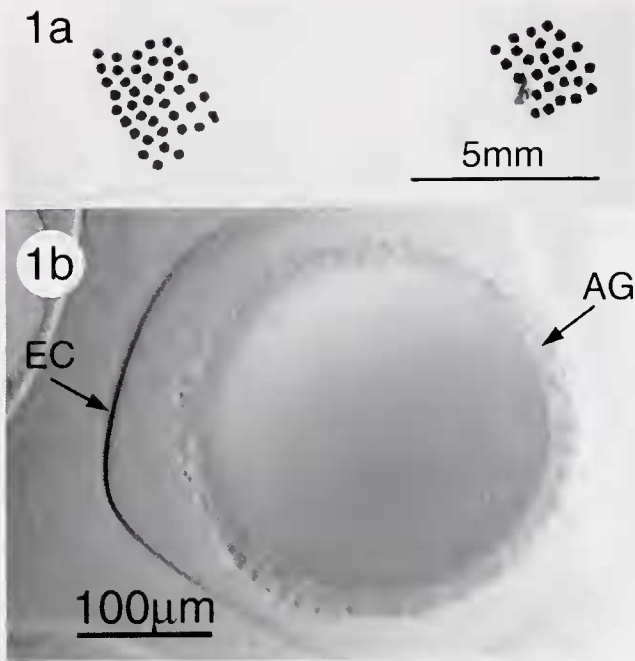
Figure 1. Eggs of *Epimenia babai.* (a) A pair of egg sheets (3 × 3 mm). Each sheet contained 20 to 50 fertilized eggs. (b) A fertilized egg (~250 μm in diameter) in a single egg capsule (EC) membrane. Numerous accessory granules (AG) were observed within the capsule.

are degenerated and lost during the transformation into the adult body form. Live larvae were incubated for 10 min in acridine orange (100 μg/ml) + ethidium bromide (100 μg/ml) in seawater. The larvae were washed in clean seawater for an hour and, while alive, were examined on a glass slide under a fluorescence microscope with a UV filter set.

The acridine orange intercalates into the DNA of live cells with intact membranes, and the cells fluoresce bright green. As apoptosis progresses, ethidium bromide enters the cells, and they fluoresce bright orange. Late apoptotic cells have a uniform bright orange color (Martin *et al.,* 1996).

Results

Reproduction and spawning

Three individuals of *Epimenia babai* laid approximately 20,000 eggs over 90 consecutive nights in the laboratory. One individual that was isolated for more than 90 days laid viable eggs. Sperm transfer was not observed.

Fertilized eggs were laid in pairs of square gelatinous sheets (3 × 3 mm) containing a single layer of 20–50 eggs (Fig. 1a). One individual laid one to four pairs of egg-sheets every night, one pair at a time, at intervals of about an hour. The egg sheets were always laid in pairs, presumably one from each of the paired gametoducts that open into the mantle cavity at the posterior end of the animal. At the time

of deposition, the fertilized eggs were always at the single-cell stage. No brooding was observed.

Early development

The early development of *E. babai* was followed using both scanning electron and light microscopy. Development was slower at 16 °C than at room temperature (26 °C) (Table 1). However, hatching time observed at 16 °C was the same as at 26 °C, with larvae reared at 16 °C hatching at a much earlier stage of development than those at 26 °C. The lower temperature did not cause any abnormalities in further development.

Observations of early embryogenesis are generally consistent with those of Baba (1940). The eggs are oval at first and become spherical within 30 min at a diameter of 250 μm. Each egg is enclosed in a tough transparent egg capsule 300–350 μm in diameter (Fig. 1b). The eggs are opaque yellow, presumably from high yolk content, and were surrounded by numerous accessory granules. Formation of two polar bodies, as reported by Baba (1938, 1940, 1951), was not observed.

Cleavage is spiral, unequal, and asynchronous (Figs. 2–9). First cleavage was meridional (= parallel to animal-vegetal axis) and unequal (Fig. 3). Just before the first cleavage was completed, the first polar lobe protruded from the vegetal side of the embryo (Fig. 3). The polar lobe appeared whiter and less yolky than the rest of the contents of the CD cell. The first polar lobe was soon absorbed by one of the two blastomeres, making this blastomere (CD) slightly larger than the other (AB).

Second cleavage was also meridional and unequal, forming blastomeres A–D. Cleavage was slightly asynchronous, with the larger blastomere (CD) dividing slightly later than the AB cell (Figs. 4, 5). The cross-furrow of the four blastomeres was visible but indistinct (Fig. 4). A second polar lobe protruded from the vegetal region during CD

Table 1

Approximate timing of early development of Epimenia babai *at 16–18 °C and ambient temperature (26 °C)*

Developmental stage	16–18 °C	26 °C
Spawning	0 h	0 h
2-cell	2 h	1 h
4-cell	4 h	2.5 h
8-cell	6 h	3.5 h
16-cell	8 h	4.5 h
32-cell	10 h	5.5–6 h
Blastula	15 h	7–9 h
Gastrula	20–24 h	10–13 h
Trochophore	48 h	24 h
Hatching	3 d	3 d
Metamorphosis	11–13 d	5–7 d
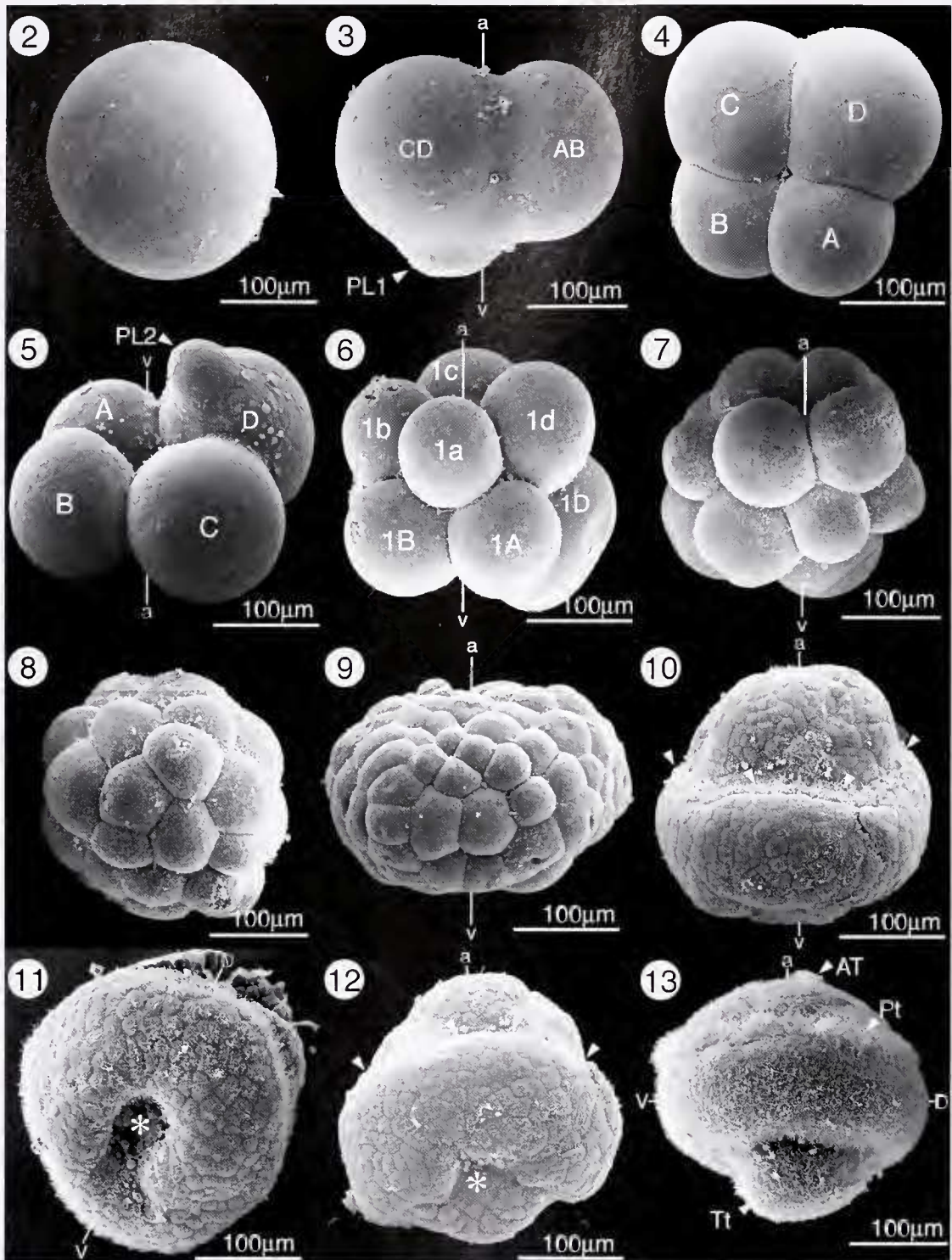
division. Within 30 min. it was absorbed by the D blastomere (Fig. 5), making it the largest of the four.

Third cleavage was equatorial (= perpendicular to the animal-vegetal axis), resulting in four micromeres at the animal pole (1a–1d); the four macromeres remained at the vegetal pole (1A–1D) (Fig. 6). The chirality of spiral cleavage displaced the four micromeres dextrally (clockwise from the animal pole) relative to the four macromeres. Cell 1D seemed to be slightly larger than the rest of the macromeres, but the difference was not substantial. Cleavage

continued to be slightly asynchronous by alternation of chirality: the fourth cleavage was sinistral (anti-clockwise from the animal pole), producing 16 cells (Fig. 7); and the fifth cleavage was dextral, producing 32 cells (Fig. 8).

The embryos reached 64 cells about 15 h after they were laid and became flattened animal-vegetally, forming a blastula (Fig. 9). Further cleavage stages until gastrulation were difficult to follow owing to the asynchronous nature of cleavage.

Within 24 h after oviposition, while the embryos were still within the capsule, gastrulation occurred at the vegetal pole by means of both epiboly and invagination (Figs. 10, 11). Cells between the blastopore and presumptive prototrochal cells thickened to form a broad lip around the vegetal side of the gastrula (Fig. 10). Patches of short cilia covered the surface of the gastrula. Cells within the blastopore proliferated to form an outgrowth of a definitive ectodermal bud beneath. From this elongation of ectoderm, the blastopore diminished and migrated toward the future ventral side of the larva (Fig. 12).

Two days after oviposition, the presumptive apical tuft and prototroch on the apical region, and the telotroch at the caudal end of the ectodermal trunk, became evident (Figs. 13, 27a). The unciliated ectodermal trunk bent slightly towards the ventral side.

Larval development

Three days after oviposition, the larvae hatched and swam forward and spirally, using the prototroch, until they reached metamorphosis 9 to 13 days after oviposition (7- to 11-day-old larvae) (see below). It is not certain whether the telotroch was directly involved in swimming or not. The larvae did not seem to be phototactic.

Larvae aged 1 to 3 days. The free-swimming larvae had three distinct body regions: apical cap, trunk, and caudal region (Figs. 14a, 27b). The entire pre-oral sphere of the larvae, the apical cap, was completely ciliated and was divided into a pretrochal and a post-trochal region by a prominent row of compound prototroch cilia at its equator (Figs. 14a, b, 16, 17, 27b). The pretrochal hemisphere of the apical cap originated most larval structures, such as the apical tuft, also composed of compound cilia (Figs. 14a, 15), and secretory globules (Figs. 17, 21a, b; morphology discussed below in detail). Cerebral ganglial depressions formed on the pretrochal hemisphere of the apical cap, and they persisted through metamorphosis (Figs. 14a, 17, 19a, 22a, b, 23a, b, 24a, 27, 28; development discussed below in detail).

The trunk region of the larvae was unciliated. The trunk gave rise to definitive ectodermal structures, such as cuticle and epidermis. A ciliated stomodeum formed on the ventral side of the unciliated ectodermal trunk directly below the apical cap (Figs. 14b, 20a, b, 27b, 28a).

The caudal region was covered with short cilia. Globules, similar to those of the pretrochal hemisphere of the apical cap, were present (Figs. 14b, 18). The cilia at the periphery and the center of the caudal region were composed of compound cilia, and the cilia were longer (*ca.* 15 μm) than on the flat bottom surface (*ca.* 10 μm) (Figs. 14b, 18).

Larvae aged 4 to 6 days. The post-trochal region of the apical cap narrowed and the cerebral depressions became deeper as the trunk grew longer (Figs. 19, 27c). A wide midventral longtitudinal band (15–20 μm across) of the trunk region of the larvae became ciliated, forming a foot (Figs. 19, 27b). At the animal end of the long foot, a pedal pit formed sharp-ended cilia, longer (3–4 μm) than the cilia on the foot, which had blunt ends (1–2 μm) (Fig. 19b). Epidermal papillae formed covering the entire trunk region (Fig. 19a, morphology discussed later in detail).

Larval morphology

Secretory organs. The function of the globules that covered the pretrochal hemisphere of the apical cap and the entire surface of the caudal region (Figs. 14a, b, 17, 18)

Figures 2–13. Scanning electron micrographs (SEM) of the early embryological development of *Epimenia babai* before hatching. Animal vegetal axis (a–v) and dorsal ventral axis (D–V) are indicated. 2. A fertilized egg just after spawning; polar bodies not detected. 3. The beginning of first cleavage (lateral view). The first polar lobe (PL1) has protruded from the vegetal side as the two cells (AB/CD) divide meridionally. The polar lobe was then absorbed by the CD cell, making it larger than AB. 4. Animal view of the 4-cell embryo (A–D). 5. Vegetal view of the 4-cell (A–D) embryo. The second polar lobe (PL2) protruded from the vegetal side of the D cell, and was soon absorbed by the D cell. 6. Lateral view of the 8-cell stage (1a–1d, 1A, 1B, 1D). 7. Lateral view of 8- to 16-cell stage. 8. Animal view of 16- to 32-cell stage. 9. Lateral view of 64-cell to blastula stage. The embryo has flattened in animal-vegetal direction. 10. Lateral view of gastrula. The arrowheads indicate ciliated presumptive prototrochal cells. The entire embryo is covered with short microvilli. 11. Vegetal view of the gastrula showing the blastopore opening (*). 12. Lateroventral view of embryo showing proliferation of the unciliated ectodermal cells dorsal to the blastopore. This outgrowth of the cells has shifted the ventral opening of the blastopore (*) towards the ventral side of the larva. 13. Tip of the outgrowth has become ciliated and forms the presumptive telotroch. The presumptive apical tuft and prototroch are apparent on the apical cap. The trunk is bent slightly towards the ventral side. a, animal pole; AT, apical tuft; D, dorsal; Pt, prototroch; Tt, telotroch; v, vegetal pole; V, ventral.
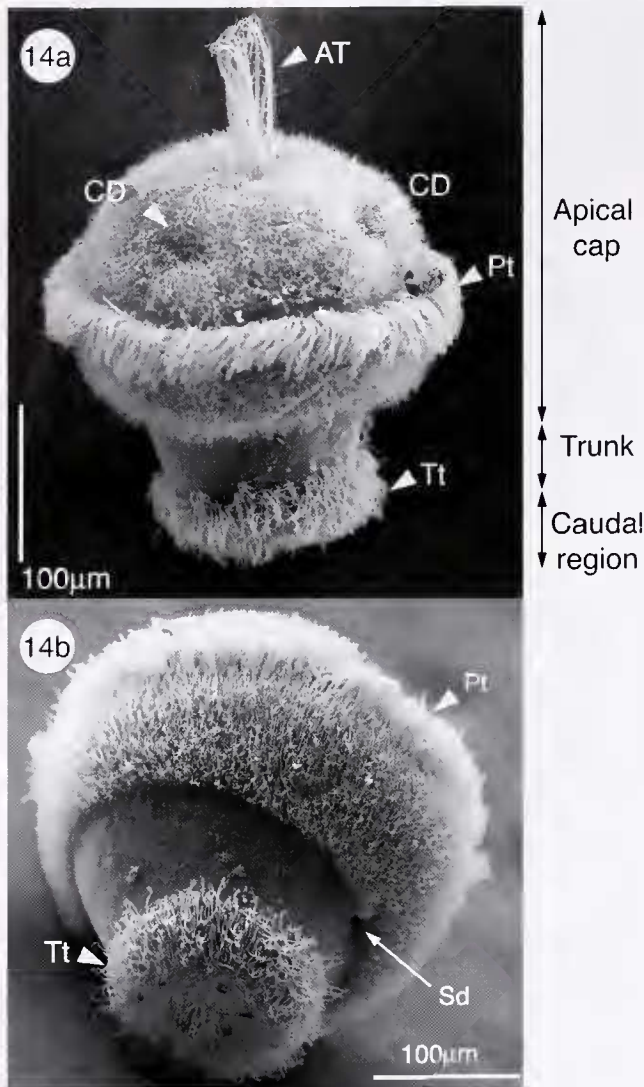
Figure 14. Larvae after hatching from the egg capsule (3 days after ovipositon, 1-day-old larvae). (a) Ventral view of the larva with three distinct body regions: ciliated pre-oral apical cap, unciliated trunk, and ciliated caudal region. (b) Vegetal (caudal) view of the trochophore larva showing the small invagination of the stomodeum on the ventral side of the trunk. AT, apical tuft; CD, cerebral depression; Pt, prototroch; Sd, stomodeum; Tt, telotroch.

appears to be secretory, because the cilia-free membranes covering the globules were often ruptured (Fig. 21a, b). The globules appeared iridescent under the light microscope. The globules disappeared at metamorphosis, as the apical cap and the caudal region were withdrawn into the trunk (Fig. 24b).

One to two hundred small epidermal papillae appeared on the trunk (Figs. 19a, 23a, 24b). Like the globules mentioned above, these papillae are suspected to be secretory in function. Unlike the globules, the papillae persisted after meta-

morphosis (Figs. 24b, 25a). Protonephridia were not observed in the light micrographs of semithin sections.

Nervous system. The cells underneath a pair of depressions on the pretrochal region of the apical cap on the future ventral side of the adult (Figs. 14a, 17, 19a, 22a, 23a, b, 24a, 27, 28) became internalized to differentiate into definitive cerebral ganglia (Fig. 22b). These depressions were free of white globules, but the surface ciliation was not different from that of the rest of the apical area (Figs. 17, 22a). Pedal ganglia arose (Fig. 22b); however, the process by which they were formed is uncertain. Development of terminal sense organs was not apparent, although may have been present.

Metamorphosis

Larvae aged 7 to 8 days: beginning of metamorphosis. In spite of active ciliary movement of the prototroch and apical tuft, larvae at this point were no longer able to swim, and they sank to the bottom of the culture jars. The unciliated trunk grew longer, the epidermal papillae increased in number and size, and presumptive spicules formed (Fig. 23c, d). Spicules seemed still to be under the cuticle just along the foot groove and beneath the apical cap (Fig. 23). Although the post-trochal region of the test narrowed as the trunk grew, lengthening of the trunk appeared to be due to proliferation of definitive ectodermal trunk cells and not to any material supplied by the larval test (Figs. 23a, 27; later discussed in the programmed cell death determination of the test). Larval structures, such as the apical tuft and telotroch, were diminished. The apical cap and the prototroch occasionally became withdrawn into the trunk (Figs. 23b, 27d, e, 28b).

Larvae aged 9 to 11 days: completion of metamorphosis. The trunk was now completely covered with epidermal papillae and spicules that extended well beyond the cuticle (Fig. 24a). The whole caudal region and the cap became enclosed within the trunk and covered by the posterior and anterior extensions of definitive ectoderm (Fig. 24b).

The post-metamorphic juveniles crawled on the ciliated foot located in the midventral pedal groove. A rudiment of the adult anterior atrium developed as an invagination above the mouth. In adults, the invagination was often open, with the anterior end and half the length of the entire body raised above the substrum. The juveniles seemed to be sensitive to vibration, but not to light.

The juveniles grew more transparent as the original yolky yellow color became internally restricted. The animals lived for up to 1 month without any particulate food source, and the gut was not differentiated in histological sections during the period.

Development of hollow spicules. Juvenile spicules are different from adult spicules in shape and distribution. The spicules of adults are solid and bladelike along the foot
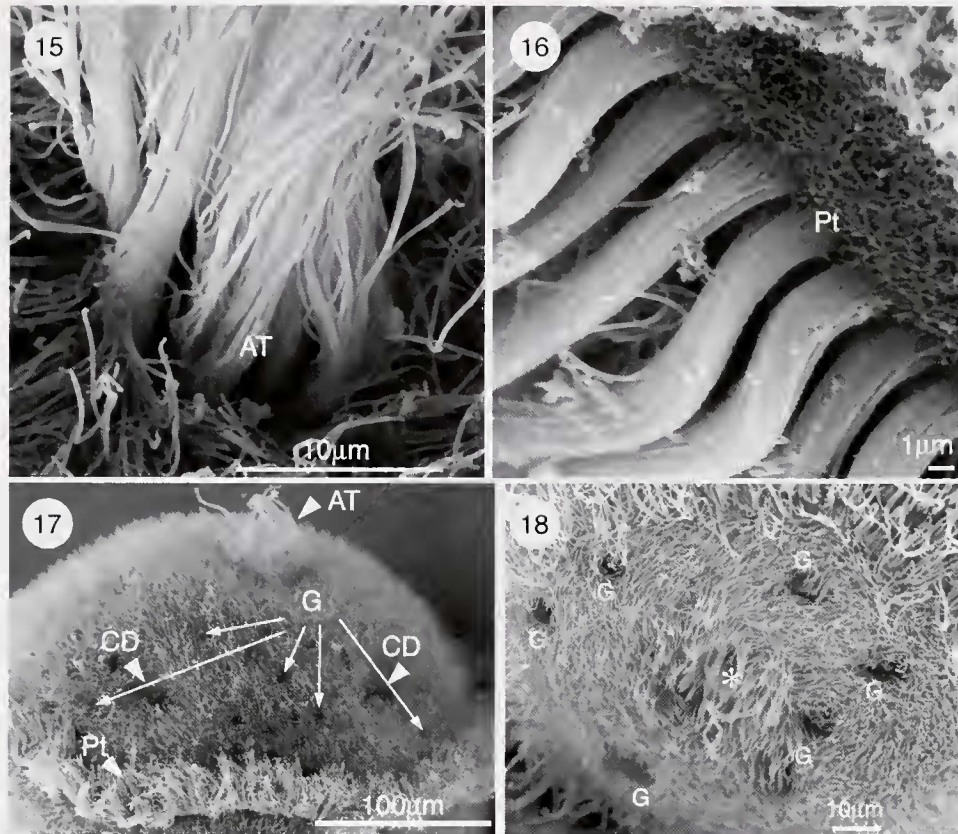
Figures 15–18. Larval structures of 1- to 3-day-old larvae. 15. Apical tuft composed of several bundles of compound cilia. 16. Compound cilia of prototroch. 17. Ventral view of the apical cap. The two lateral depressions (CD) mark where cells are proliferating internally to form the cerebral ganglia. Unciliated globules are scattered between and on both sides of the depressions. 18. Bottom surface of the caudal region. The entire surface is ciliated except for scattered unciliated globules. The cilia at the periphery and in the center of the caudal region (*) are longer than the rest of the cilia on the caudal surface. AT, apical tuft; CD, cerebral depression; G, globules; Pt, prototroch.

groove and near the mouth, but the rest of the body is covered by hollow, needlelike spicules forming a criss-crossed meshwork within the cuticle. In juveniles, however, flat bladelike spicules were distributed evenly over the entire body along with upright and needlelike spicules (Fig. 25a, c). The juvenile pedal spicules were flat and bladelike as in the adults (Fig. 25b, c), and the head region also was covered with flat, bladelike spicules (Fig. 25d).

The development of hollow spicules in early juveniles was observed for the first time in aplacophorans (Fig. 26) by observing within the cuticle of live larvae on a glass slide using polarized light microscopy. The first part of the spicules to be secreted was the solid distal tip (Fig. 26a). The spicules continued to grow at the proximal end, becoming hollow with an open end (Fig. 26b). The proximal end finally closed to form a closed-ended hollow spicule with solid base (Fig. 26c). This type of hollow spicule formation is known to continue throughout the growth of adult neomenioids (Hoffman, 1949).

Morphology and fate of apical cells

Hoechst nuclear staining. Hoechst nuclear staining showed that cells of the apical cap were much larger than cells of the trunk region and stayed relatively constant in size (Fig. 27). It also showed that the number of the cells in the apical cap decreased as the apical cap degenerated, while the cells in the trunk region decreased in size and increased in number through proliferation (Fig. 27).

Programmed cell death determination. Cell viability and apoptotic index of apical cells were determined using programmed cell death (PCD) staining. At a stage as early as the first day of hatching, the entire pre-oral region of the embryos, including the prototrochal cells, stained orange (Fig. 28a), thus indicating PCD. The cerebral ganglial depressions, however, remained green and thus viable (Fig. 28a). The definitive ectodermal trunk also remained green and viable (Fig. 28a). The caudal region stained orange (Fig. 28a), indicating that the caudal cells also were degenerating.
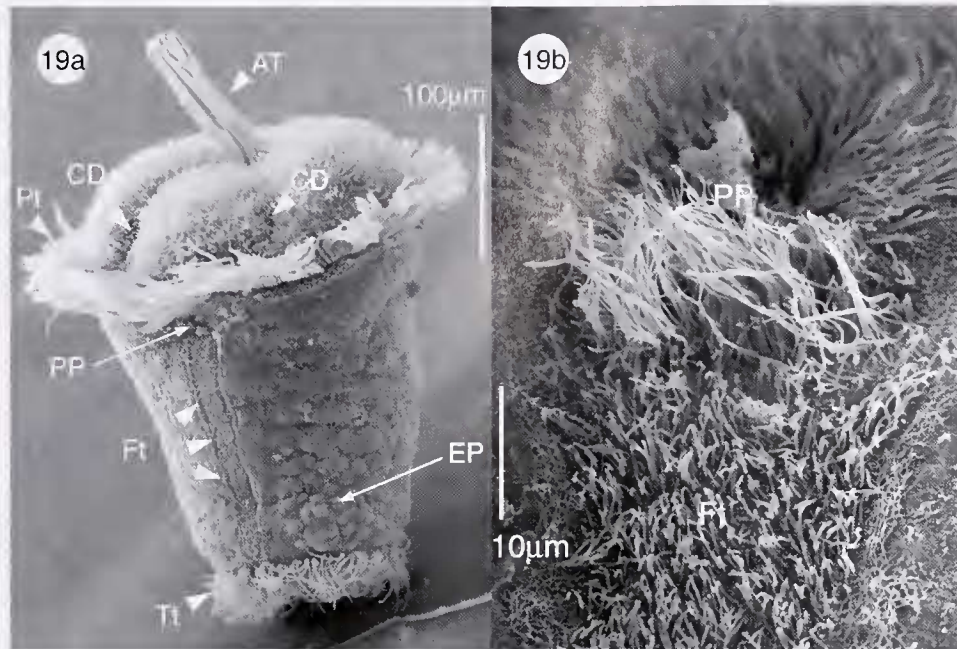
Figure 19. Larvae aged 4 to 6 days. (a) Ventral view. The ciliated foot has formed midventrally on the trunk, and the pedal pit has formed at its apical end. Epidermal papillae are beginning to form on the trunk. The post-trochal region of the apical cap is diminishing as the trunk region elongates. (b) Cilia in the pedal pit are longer (3–4 μm) and more slender than those on the foot (1–2 μm). AT, apical tuft; CD, cerebral depression; EP, epidermal papilla; Ft, foot; PP, pedal pit; Pt, prototroch; Tt, telotroch.

The pre-oral region of larvae at later stages, 5 to 7 days old, stained in brighter orange to red as it became withdrawn into the definitive ectoderm (Fig. 28b), indicating that these cells entered later stages of apoptosis. The caudal telotroch, partially withdrawn, also stained brighter orange. The elongated ectodermal trunk still remained green, indicating viable cells, but the epidermal papillae stained in orange to red, presumably because they are secretory and undergoing rapid turnover and cell death.

Discussion

Observations and remarks on reproduction and development

As observed in this study, reproduction and spawning in *Epimenia babai* were mostly consistent with Baba's descriptions (1938, 1940, 1951). *E. babai* is hermaphroditic, and although the time of sperm transfer is not known, fertilization is assumed to have occurred internally while the posterior ends of animals were entwined. This behavior has also been reported in *E. australis* (Scheltema and Jebb, 1994). However, an observation that one individual isolated for more than 90 days laid viable fertilized eggs suggests either that *E. babai* can self-fertilize, or that sperm were held for that period in the seminal receptacles.

Although brooding was not observed in *E. babai* during this study, the capacity to keep embryos within the posterior

mantle cavity in fast water currents cannot be refuted. Brooding of larvae in the posterior mantle cavity was previously reported in *E. babai* (Baba, 1938, 1951), as well as in *E. australis* (Scheltema and Jebb, 1994), *Halomenia gravida* (Heath, 1918), and some perimeniid (= "pruvotinid") neomenioids (Thiele, 1913; Salvini-Plawen, 1978). During this study, *E. babai* individuals tightly curled their posterior part around the alcyonarian corals, thus pressing closed the mantle cavity opening and keeping their eggs within the mantle cavity. In their natural habitat, *E. babai* and other species of *Epimenia* live and feed on the alcyonarian corals that prefer strong swift water currents (Baba, 1938, 1940, 1951; Salvini-Plawen, 1972; Salvini-Plawen and Benayahu, 1991; Scheltema and Jebb, 1994). Therefore, brooding may take place in the natural habitat.

Homology of tests among neomenioid aplacophorans

On the basis of similarity in cell type, ontogeny, ciliation pattern, and cell fate of the apical cap, the larva of *E. babai* is here proposed to have a test-like apical cap with cells similar to the test cells of larvae of other neomenioid aplacophorans: *Neomenia carinata*, *Nematomenia banyulensis*, and *Rhopalomenia aglaopheniae* (Pruvot, 1890, 1892; Thompson, 1960) (Fig. 29a). The apical cap of *E. babai* and the tests of the other neomenioids are composed of large yolky cells that are larval in fate and pre-oral in origin, are
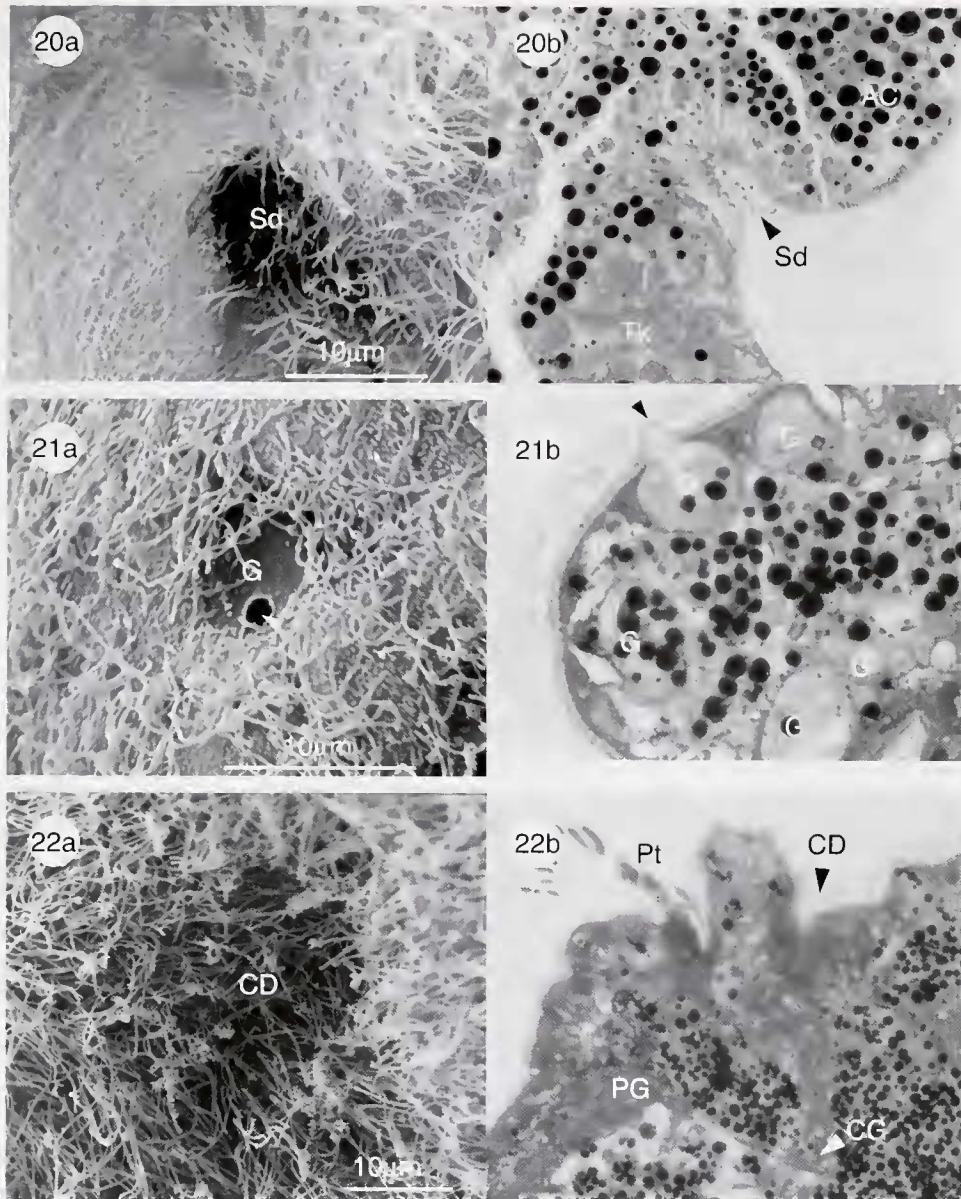
Figures 20–22. Development of stomodeum, globules, and cerebral depression of 1- to 3-day-old larvae. 20a. SEM image at the first sign of the stomodeal invagination on the trunk just beneath the apical cap. 20b. Light microscope image of a histological section through the ciliated stomodeum. 21a. SEM image of a globule (Figs. 17, 18) with an unciliated surface and a hole (arrowhead) that remained after the globule had ruptured. 21b. Light microscope image of a histological section of a globule as it ruptured (arrowhead). 22a. SEM image of the surface of one of the two cerebral depressions on the apical cap. The surface ciliation of the depression was not different from the surrounding area. 22b. Light microscope image of cells beneath the depression (arrowhead) that have proliferated into a cerebral ganglion, which connects to a pedal ganglion. AC, apical cap; CD, cerebral depression; CG, cerebral ganglion; G, globule; Sd, stomodeum; PG, pedal ganglion; Pt, prototroch; Tk, trunk.

covered with uniform short cilia, have a row of prototrochal cells and an apical tuft, and have cerebral ganglia depressions that persist through metamorphosis. The "test" of *E. babai* is less obvious because its cells are much smaller than those of other neomenioid tests, thus leaving the developing definitive ectodermal trunk underneath more exposed (Fig. 29).

The apical cap cells of the *E. babai* larvae are entirely apoptotic and, like the tests of other neomenioid aplacophorans, presumably do not contribute to development of any definitive adult structures except for the cerebral ganglia. The tests of *Nematomenia banyulensis* and *Rhopalomenia aglaopheniae* were said by Pruvot (1890, 1892) to be cast off and discarded at metamorphosis. The test cells of
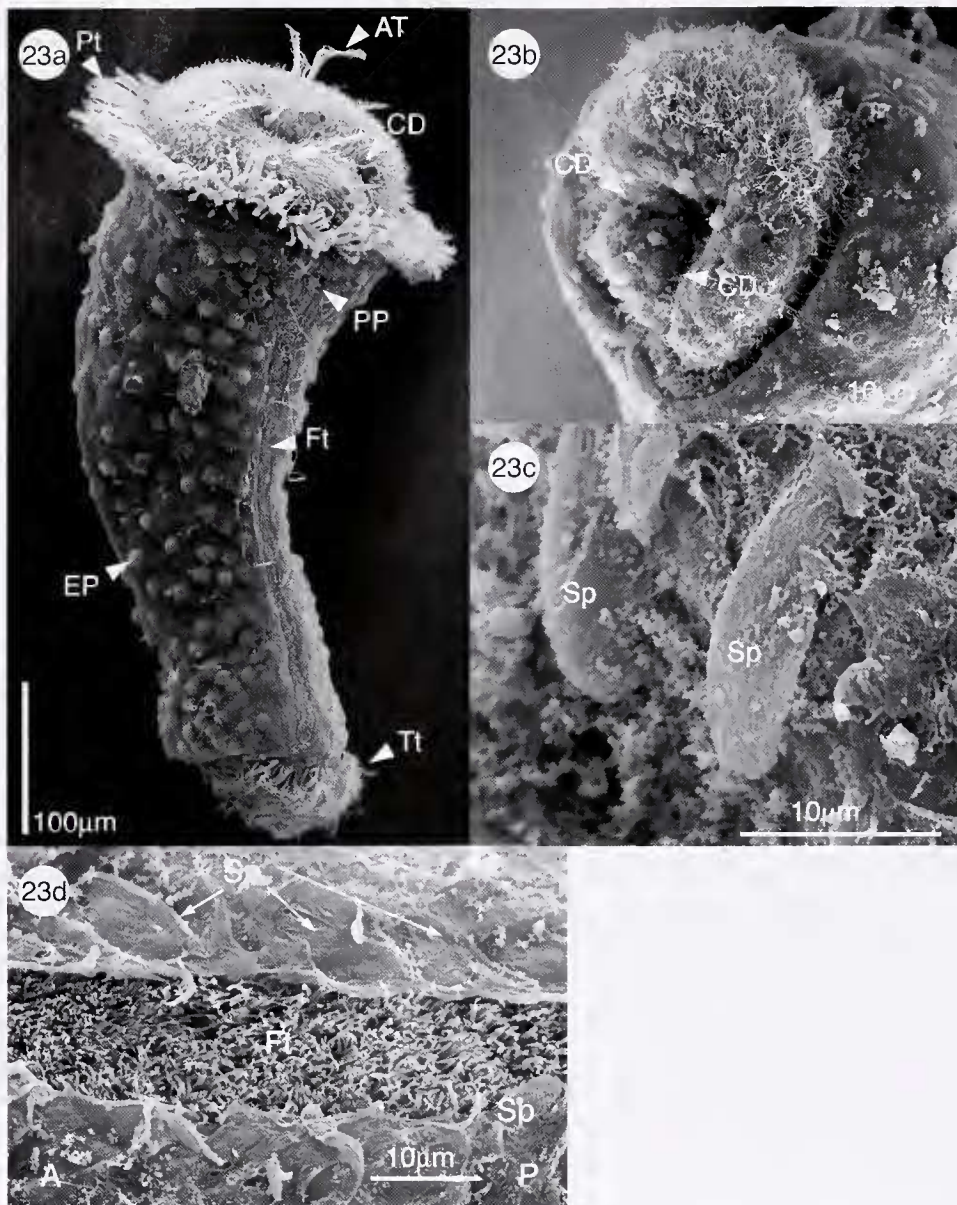
Figure 23.   Beginning of the gradual process of metamorphosis (7- to 8-day-old larvae). (a) The post-trochal region of the apical cap has completely disappeared. The trunk has become covered with numerous large epidermal papillae. (b) A larva with its apical region withdrawn into the trunk. (c) Aragonitic larval spicules, restricted to the trunk region, have started to appear underneath the cuticle. (d) Regularly arranged spicules underneath the cuticle beside the foot. AT, apical tuft; CD, cerebral depression; EP, epidermal papilla; Ft, foot; Sp, spicule; PP, pedal pit; Pt, prototroch; Tt, telotroch.

Neomenia carinata are absorbed by the animal at metamorphosis, as are the apical cap cells of *E. babai*; they degenerate and in *N. carinata* are known to be the main food reserve of post-larvae (Thompson, 1960). The fate of the cells in the brooded larvae of *Halomenia gravida* is not clear, although Heath (1918) observed in histological sections that they become reduced in size at later stages, indicating resorption.

There are some differences among neomenioid apla-

cophoran species in the number of "test" cells and in number of pairs of cerebral ganglia that proliferate from the test (Table 2). Test cells are arranged in six regular tiers of cells in *Nematomenia banyulensis* and *Rhopalomenia aglaopheniae* (Pruvot, 1890, 1892) and in five regular tiers of cells in *Neomenia carinata* (Thompson, 1960), whereas the apical cap cells of *E. babai* are numerous and irregularly arranged. The number of cerebral ganglia invaginations also varies: there is one pair in *E. babai*, *Nematomenia banyulensis*
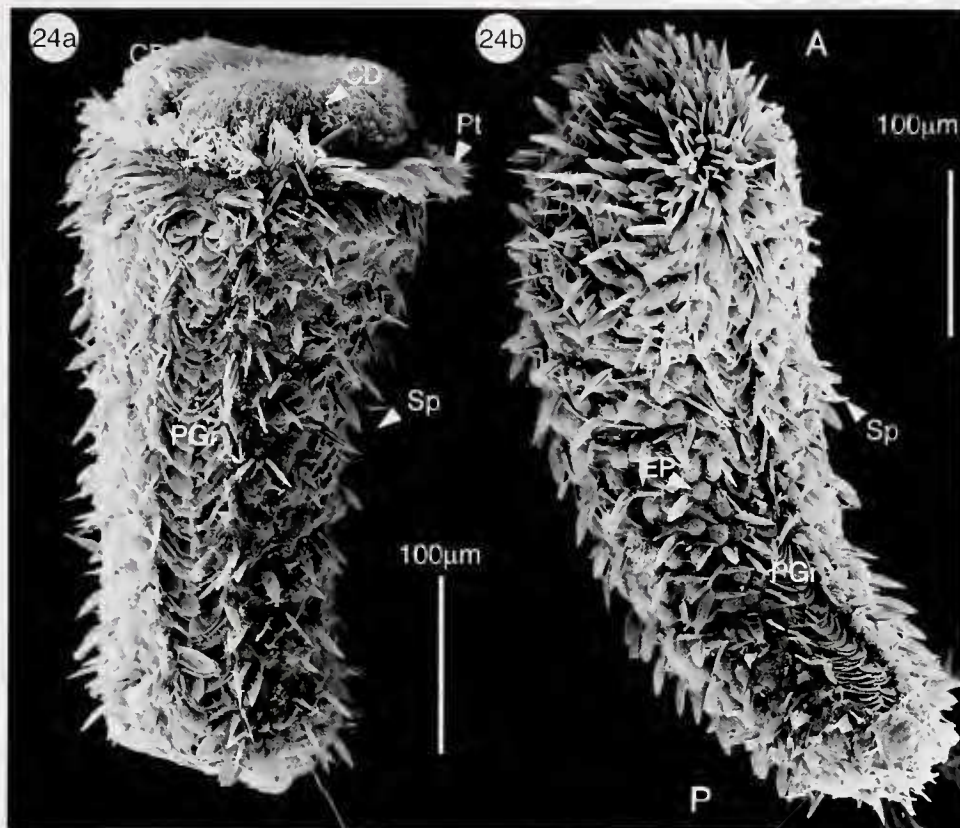
Figure 24. Larvae during the completion of metamorphosis (9- to 11-day-old larvae). (a) The larva covered by spicules on the trunk. The apical cap has diminished, and the apical tuft is being resorbed at the apical end. (b) Post-metamorphic juvenile. The apical cap and caudal regions have been completely engulfed by the trunk, leaving the trunk as the only definitive juvenile structure. A, anterior; CD, cerebral depression; EP, epidermal papilla; Sp, spicule; P, posterior; PGr, pedal groove; Pt, prototroch.

and *R. aglaopheniae* (Pruvot, 1890, 1892), whereas there are three pairs in *Neomenia carinata*, the third pair corresponding to the pedal ganglia (Thompson, 1960). In *Nematomenia banyulensis* and *R. aglaopheniae*, the pedal ganglia proliferate and split off from the cerebral ganglia internally (Pruvot, 1890, 1892). The process of pedal ganglia formation was not clear for *E. babai*.

Phylogenetic affinities of pericalymma larvae

Tests or test-like structures also occur in other molluscan taxa. Diverse forms of tests are described from six protobranch species: *Acila castrensis* (Zardus and Morse, 1998), *N. delphinodonta* (Drew, 1901), *Nucula proxima* (Drew, 1899a), *Solemya reidi* (Gustafson and Reid, 1986), *S. velum* (Gustafson and Lutz, 1992), and *Yoldia limatula* (Drew, 1899b). Protobranch bivalve tests are composed of large yolky cells, and the tests engulf the larvae entirely (*N. delphinodonta, S. reidi, S. velum,* and *Y. limatula*) or only partly (*A. castrensis* and *N. proxima*). The protobranch tests are larval and are cast off and ingested through the mouth at metamorphosis. The ciliation pattern of protobranch bivalve

larvae is diverse: *A. castrensis, N. proxima,* and *Y. limatula* have an apical tuft and three rows of ciliary bands, whereas *N. delphinodonta, S. reidi,* and *S. velum* have no apical tuft and no ciliary bands. The tests of *A. castrensis, N. delphinodonta, N. proxima,* and *Y. limatula* are composed of five rows of cells, whereas the tests of *S. reidi* and *S. velum* are composed of nine. The caudal organ also seems to be diverse among protobranchs: *A. castrensis* and *S. reidi* have a ciliated caudal organ, *N. proxima* has an unciliated caudal organ, and *S. velum* and *Y. limatula* have no caudal organ.

Test-like structures are also found among trochophore larvae of scaphopods, described as having an apical tuft and three or six ciliary bands (Lacaze-Duthiers, 1856; Geilenkirchen *et al.,* 1970; Guerrier *et al.,* 1978; Wanninger and Haszprunar, 2001). No ciliated caudal structure has been reported. The test of scaphopods, in later developmental stages, becomes pushed anteriorly to form a velum (Lacaze-Duthiers, 1856). The scaphopod velum has been homologized with the lamellibranch bivalve velum. However, the scaphopod velum functions solely in locomotion, as in the
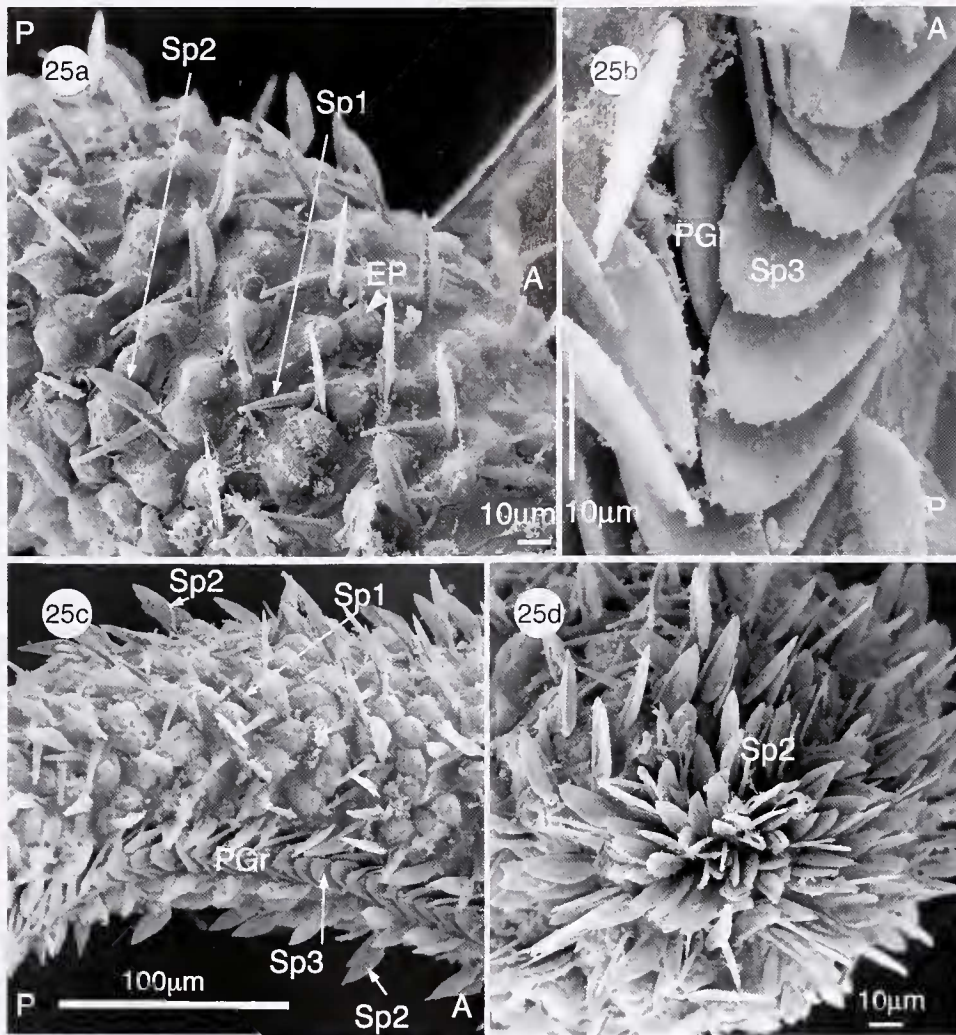
Figure 25. Three types of larval spicules right after metamorphosis. (a) Dorsal view. Two types of spicules are equally abundant over the entire body of the juvenile: hollow upright spicules (Sp1) lie against the body, while bladelike solid spicules (Sp2) project outwards from the body. (b) The third type of larval spicule (Sp3) is broad and solid, forming a single row on each side of the pedal groove. (c) Ventral view. (d) Anteriormost region covered with Sp2-type spicules. A, anterior; EP, epidermal papilla; P, posterior; PGr, pedal groove; Sp1, spicule type 1; Sp2, spicule type 2; Sp3, spicule type 3.

pericalymma tests, whereas the lamellibranch bivalve velum also functions in feeding, and the homology is not certain.

Polyplacophorans (Grave, 1932; Eernisse and Reynolds, 1994), archaegastropods (Patten, 1886; Kessel, 1964), and chaetoderm aplacophorans (Nielsen, 2001) have trochophore larvae with a ciliated circular velum. Polyplacophoran vela are composed of one to three rows of prototrochal bands (Grave, 1932; pers. obs.). The ventral side of the ciliated apical region of polyplacophorans becomes absorbed at metamorphosis, while the dorsal side of the apical region gives rise to adult features such as the anteriormost shell valve and spicules (Grave, 1932). The archaegastropod vela also have three rows of prototrochal bands,

which function in both locomotion and feeding. There seem to be several rows of ciliary bands on the chaetoderm aplacophoran vela; however, lack of complete developmental descriptions of the group limits speculation.

The question of the ancestral larval type among molluscs has been important in many discussions of their origin and diversification. The pericalymma of neomenioid aplacophorans and protobranch bivalves has been regarded as plesiomorphic by some authors (e.g., Drew, 1901; Thompson, 1960; Salvini-Plawen, 1972, 1973, 1980), although the trochophore has been generally considered plesiomorphic (Haszprunar et al., 1995; Rouse, 1999) and the pericalymma as derived. It has also been suggested that the lamellibranch velum could have arisen from the coalescence of perica-
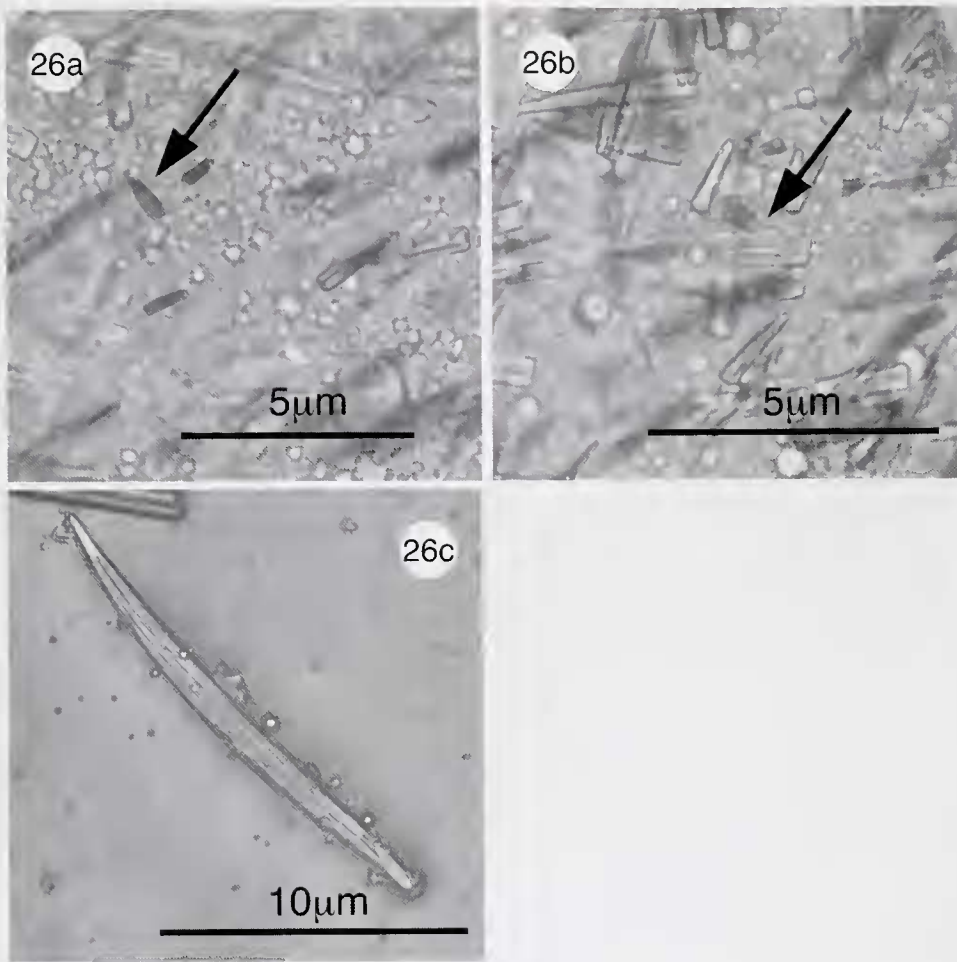
Figure 26. Developmental sequence of hollow spicules in *Epimenia babai* juveniles. Spicule begins as a solid tip (a), develops to a hollow, open spicule (b), and finally becomes a hollow, closed spicule (c).

lymma test (Drew, 1899b, 1901). However, there are as yet neither sufficient morphological data nor any cell lineage data to resolve the question of homology among the tests and homology of the tests to the velum.

Trochophore larvae seem to have gone through many modifications, not only within the clade Mollusca, but also in other spiralian taxa. Test-like structures are also found among lecithotrophic larvae of non-molluscan spiralians: the "serosa" of the sipunculid *Sipunculus nudus* (Rice, 1988); the "endolarva" of the polychaete *Phyllodoce mucosa* (Cazaux, 1970) and other Phyllodocidae spp. (Dawydoff, 1959); the "Iwata's larva" of a heteronemertean (Korschelt, 1936; Iwata, 1958; Jägersten, 1968); and the lecithotrophic nemertean larvae of *Emplectonema gracile* (Delsman, 1915), *Geonemertes australiensis* (Hickmann, 1963), and *Tetrastemma candidum* (Maslakova and Malakhov, 1999).

Given that test-like structures occur across various distant taxa within Spiralia that have lecithotrophic larvae, that their tests are composed of yolk cells, and that the tests are

merely an enlarged pre-oral region of a trochophore larva, the tests could have arisen independently in each clade by adaptation to lecithotrophy. Homology among larval tests within molluscs and among spiralians and the pattern in which they evolved within the clade can probably be ascertained by more detailed developmental data analyzed within a well-corroborated phylogenetic framework. However, the phylogeny of Mollusca is controversial; cladistic analysis of a limited number of morphological characters (Salvini-Plawen and Steiner, 1996; Waller, 1998; Haszprunar, 2000) and available molecular sequence data on 18S rRNA (Winnepenninckx *et al.*, 1996) have not yet resolved the issue. Larval development of other neomenioids and the Chaetodermomorpha needs to be studied in order to understand the diversity of developmental processes among aplacophorans. In addition, cell lineage tracing would help to ascertain the exact ontogeny of the tests among molluscs and other spiralians. Furthermore, developmental descriptions of monoplacophoran larvae, which are entirely lacking, are desired.

29

a. *Epimenia babai*

apical cap
(test)

b. *Neomenia carinata*

c. *Nematomenia banyulensis*
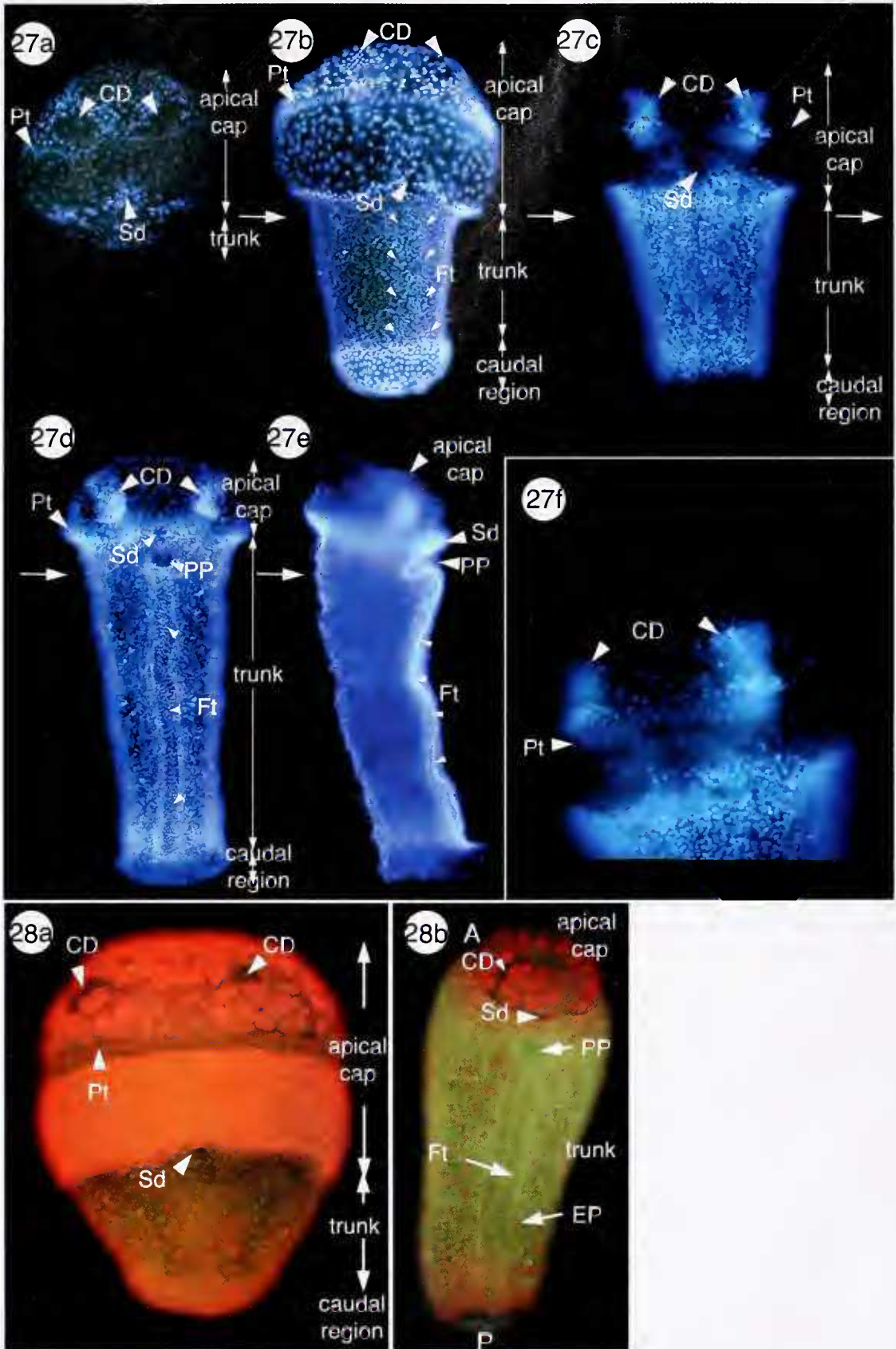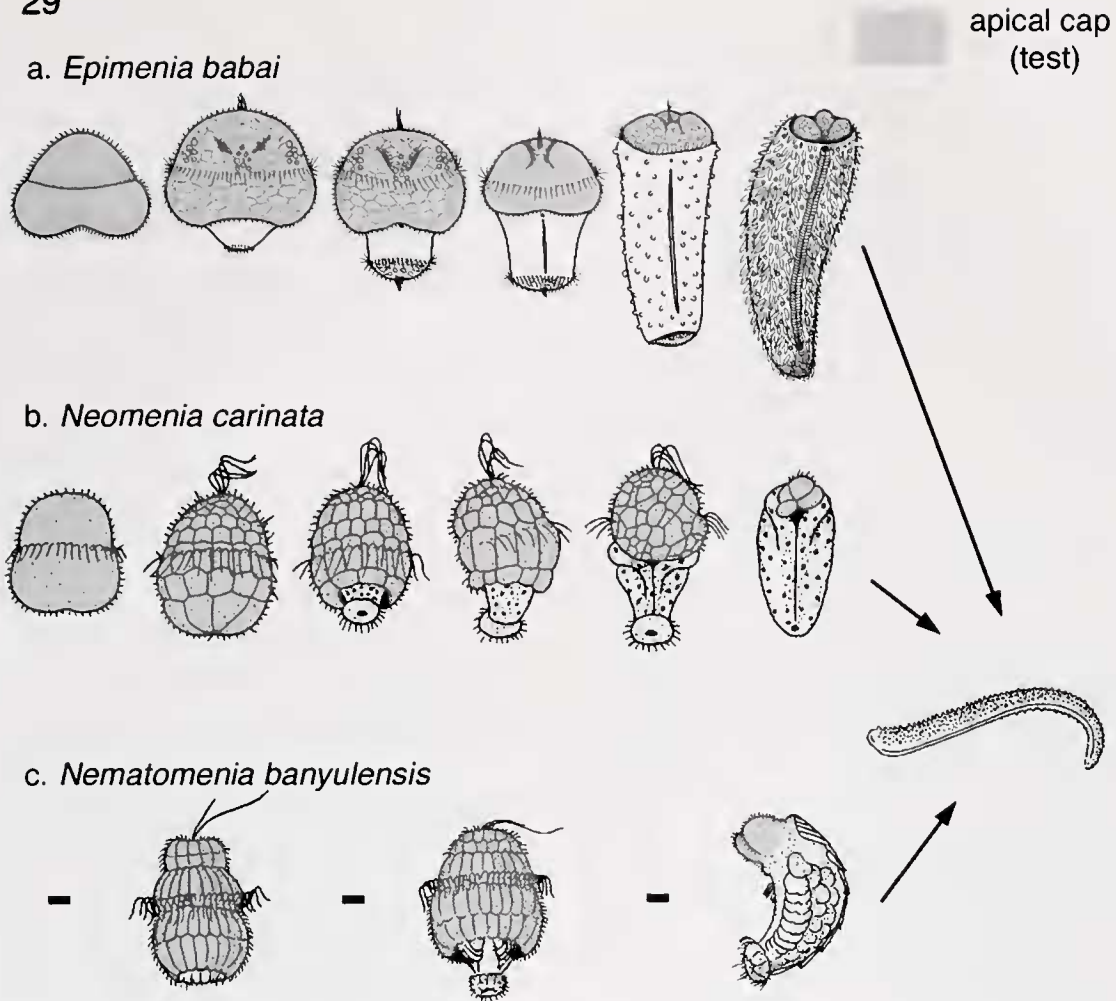
Figure 29. Developmental sequences of free-swimming larvae compared among the three neomenioid aplacophorans: (a) *Epimenia babai.* (b) *Neomenia carinata* (modified from Thompson, 1960). (c) *Nematomenia banyulensis* (modified from Pruvot, 1890). The gray shading indicates apical cap (test) structure.

Figure 27. Hoechst nuclear staining showing the development of larvae and the degeneration of large apical cap cells. (a) Ventral view of a larva right before hatching, showing apical cap and trunk (*cf.* Fig. 13). (b) Ventral view of a 3- to 4-day-old larva (*cf.* Figs. 14, 19). Cells of the apical cap are larger than cells on the trunk. Arrowheads indicate the broad ciliated foot starting to form. (c) Ventral view of a 5-day-old-larva (*cf.* Fig. 19). The size of the cells in the apical cap has not changed, whereas the cells in the trunk region have become smaller and fewer in number as they proliferated and differentiated. (d) Ventral view of a 7-day-old larva (*cf.* Fig. 23). The number of cells in the apical cap has decreased; post-trochal region of the apical cap has diminished. The cells in the apical cap still remain large. The foot narrows as the trunk elongates. (e) The lateral view of a 7-day-old larva. The apical cap is being withdrawn into the trunk. (f) Apical cap of a 5-day-old larva. The cells of the apical cap remain large, and they appear to be transparent and degenerating. CD, cerebral depression; Ft, foot; PP, pedal pit; Pt, prototroch; Sd, stomodeum.

Figure 28. Programmed cell death (PCD) of apical cap cells and caudal region of *Epimenia babai* larvae. (a) 2-day-old larva. The entire pre-oral apical cap is stained orange to red, indicating PCD, except for the cerebral depressions, which remained green, indicating viable cells. The definitive ectodermal trunk has remained green, also indicating viable cells. The caudal region also stained orange to red, indicating PCD. (b) 8-day-old larva. The apical cells indicating PCD are withdrawing into the definitive trunk. The trunk remains green and thus viable; however, the epidermal papillae are stained for PCD. A, anterior; CD, cerebral depression; EP, epidermal papillae; Ft, foot; P, posterior; PP, pedal pit, Pt, protroch; Sd, stomodeum.

Table 2

Modes of development of neomenioid aplacophorans

	Egg diameter (µm)	Mode of larval nutrient	Test Morphology						Test fate	Telotroch/ caudal organ
			Apical tuft	No. cell rows in prototroch	No. pairs of ectodermal cerebral depressions	No. rows of test cells	No. cells in test			
Nematomenia banyulensis[1]	110–120	lecithotrophic	+	1	?	6	56		cast off?	ciliated telotroch
Rhopalomenia aglaopheniae[1]	260	lecithotrophic	+	1	?	6	?		cast off?	ciliated telotroch
Halomenia gravida[2]	320	lecithotrophic (brooded)	?	?	?	?	?		?	ciliated telotroch
Neomenia carinata[3]	?	lecithotrophic	+	1	3	5	~80		resorbed	ciliated telotroch
Epimenia babai[4]	250	lecithotrophic (brooded?)	+	1	1	no regularity	>100		resorbed •	ciliated telotroch

[1] Pruvot, 1890, 1892; [2] Heath, 1918; [3] Thompson, 1960; [4] Baba, 1938, 1940, 1951, present study.

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